REMARKS

Applicants respectfully request reconsideration and reexamination of the present application in light of the foregoing amendments and following remarks. Amendments are made without disclaimer or prejudice to Applicants' rights to pursue any canceled subject matter in this or a continuing application.

1. Status of the Claims

Claims 1-58 are pending. Claims 1-12 stand allowed. Claims 13-58 are rejected.

2. Support for the Amendments

Support for a method of treating a thromboxane A_2 (TXA_2)-mediated disorder related to platelet aggregation is found in the specification, for example, at p. 19, lines 10-19; p. 21, lines 20-23; p. 27, lines 25-30; p. 45, line 13 through p. 46, line 11 (the recited compounds inhibit the binding of TXA_2 to the human platelet TXA_2 receptor).

Support for a method of treating a disorder characterized by convulsive seizures in an individual is found in original claims 37 and 38 and in the specification, for example, at p. 25, lines 3-8 (an effective amount to decrease the frequency and/or severity of seizures); Example 6 (efficacy in a tonic/clonic seizure assay).

3. Notice of Related Prosecution

The Office asserts that the present application contains subject matter related to several co-pending applications. Applications previously alleged to be related but not listed below are abandoned. The remaining allegedly related applications are co-pending and have the following status as of February 13, 2009:

a) Application Serial No. 10/578,522: Applicants filed a response and amendment dated February 6, 2009; and
b) Application Serial No. 10/461,290: an Office Action last issued in the '290 application on September 3, 2008.

4. **Restriction Requirement**

Applicants appreciate the withdrawal of the restriction requirement and rejoinder of claims 13-58.

5. **Indication of Allowable Subject Matter**

Applicants appreciate the indication that claims 1-12 are allowed.

6. **Rejections under 35 U.S.C. § 101**

Claims 13-17 are rejected under 35 U.S.C. § 101 as allegedly claiming the same invention as claims 5 and 7-12 of U.S. Patent No. 6,864,251 B2. The rejection is mooted by cancelation of claims 13-21 without prejudice or disclaimer.

Claims 13, 17, and 18 are rejected under 35 U.S.C. § 101 as allegedly claiming the same invention as claim 12 of U.S. Patent No. 6,638,928 B1. The rejection is mooted by cancelation of claims 13-21 without prejudice or disclaimer.

7. **Rejections under the Doctrine of Statutory Double Patenting**

Claims 13-17 are ***provisionally*** rejected under the judicially created doctrine of obviousness-type double patenting over U.S. Application No. 10/578,522. The rejection is mooted by cancelation of claims 13-21 without prejudice or disclaimer.

8. **Rejections under 35 U.S.C. § 112, First Paragraph (Enablement)**

I

Claims 22-25

Claims 22-32 are rejected under 35 U.S.C. § 112, first paragraph, as allegedly directed to subject matter not enabled by the specification. Applicants traverse the rejection as it applies to amended claims 22-25; the rejection is moot with respect to claims 26-32 by the cancellation of the claims without prejudice or disclaimer.

The Office purports to analyze enablement using the factors set forth in *In re Wands*, 858 F.2d 731, 736, 8 U.S.P.Q.2d 1400 (Fed. Cir. 1988). The *Wands* factors include (1) the nature of the invention, (2) the breadth of the claims, (3) the state of the prior art, (4) the predictability or unpredictability of the art, (5) the relative skill of those in the art, (6)

the amount of direction or guidance presented, (7) the presence or absence of working examples, and (8) the quantity of experimentation necessary.

(A) Nature of the invention:

The present claims are directed to a method of treating a TXA_2-mediated disorder related to platelet aggregation. The specification discloses that the allowed compounds inhibit the binding of TXA_2 to its receptor on human platelets. *See, e.g.*, Specification, p. 45, line 13 through p. 46, line 11. The binding of TXA_2 to the platelet receptor is long known in the art to start a cascade of events that results in platelet aggregation and the concomitant platelet release reaction. *See, e.g.*, Armstrong *et al.*, *Br. J. Pharm.* 79: 953-64 (1983) at p. 953, 1st col. (cited in the specification at p. 45, line 26). The skilled artisan thus expects TXA_2 receptor antagonists to inhibit platelet aggregation. *See id.*, p. 954, 1st col.; Fig. 7; p. 961, 1st col. Likewise, the skilled artisan reasonably expects compounds exhibiting antiplatelet activity to be useful in treating disorders related to platelet aggregation, e.g., myocardial infarction. *See, e.g.*, Vermylen *et al.*, *Cardio. Drugs Ther.* 6: 29-33 (1992) ("Vermylen"; cited by the Office), p. 29, 1st col.

(B) Breadth of the claims:

Accordingly, the claims are directed to a method of treating a TXA_2-mediated disorder related to platelet aggregation. The Office must apply the broadest reasonable interpretation of "treating." *See In re Cortright*, 49 U.S.P.Q.2d 1464, 1469, 165 F3d 1353, 1358 (Fed. Cir. 1999) (reversing the Office's rejection of a claim to "restoring hair growth," which the Office misconstrued narrowly as "returning the user's hair to its original state"). In the present case, the meaning of "treating" is consistent with an alleviation of a disorder. *See, e.g.*, Specification, p. 26, lines 8-11. For example, a "risk reduction," like that discussed in Vermylen, falls within the meaning of a "treatment," even if it is not a complete reduction of risk.

(C) State of the relevant art:

The Office alleges that the relevant art is unpredictable, based on "disappointing" results of earlier clinical trials with inhibitors of TXA_2 synthesis inhibitors. Office Action, pp. 12-13. First, the present compounds work by antagonizing the binding of TXA_2 to its receptor, not by inhibiting TXA_2 synthesis. Vermylen reports that TXA_2 receptor

antagonists generally inhibit platelet function more reproducibly. *See* Vermylen, p. 30, 2nd col.

Second, for the reasons above, the artisan would expect compounds that antagonize the platelet TXA_2 receptor to have some ability to treat a TXA_2-mediated disorder related to platelet aggregation, irrespective of results in clinical trials. In this regard, it is well established that the legal standard for compliance with the enablement requirement is separable from any requirement for efficacy in clinical trials. In short, whether a compound is effective in clinical trials is for the FDA—not the PTO—to determine: "Title 35 does not demand that such human testing occur within the confines of [PTO] proceedings." *In re Brana*, 34 U.S.P.Q.2d 1436, 1442 (Fed. Cir. 1995) (quoting *Scott v. Finney,* 34 F.3d 1058, 1063, 32 U.S.P.Q.2d 1115, 1120 (Fed. Cir. 1994)).

(D) Working example:

The specification provides a working example of a method of treating a TXA_2-mediated disorder related to platelet aggregation, where it discloses that the present compounds antagonize the binding of TXA_2 to the human platelet TXA_2 receptor. *See, e.g.*, Specification, p. 45, line 13 through p. 46, line 11. An *in vitro* test of a compound serves as a working example of *in vivo* efficacy, if the skilled artisan would accept the *in vitro* model as reasonably correlating to the condition to be treated. *See, e.g.*, *Brana*, 51 F.3d at 1566, 34 U.S.P.Q.2d at 1441 (reversing the PTO decision based on finding that *in vitro* data did not support *in vivo* applications); *Cross v. Iizuka*, 753 F.2d 1040, 1050, 224 U.S.P.Q. 739, 747 (Fed. Cir. 1985) (a rigorous or an invariable exact correlation is not required); *see generally* MPEP § 2164.02, "Working Example," 8th ed., revised Aug. 2006.

As set forth in Part I, 8(A), above, the skilled artisan considers *in vitro* antagonism of the platelet TXA_2 receptor to correlate reasonably with inhibition of platelet aggregation. *See, e.g.*, Armstrong, p. 953, 1st col.; p. 954, 1st col.; Fig. 7; p. 961, 1st col. Likewise, the skilled artisan reasonably expects compounds exhibiting antiplatelet activity *in vitro* to be useful in treating disorders related to platelet aggregation, e.g., myocardial infarction. *See, e.g.*, Vermylen, p. 29, 1st col. It follows that the successful *in vitro* testing of the claimed compounds constitutes a working example of the claimed method of treating a TXA_2-mediated disorder related to platelet aggregation.

(E) Additional guidance:

The specification discloses at length pharmaceutical compositions that are useful for the present methods. The disclosure also guides the selection of effective amounts, routes of administration, etc. *See, e.g.*, Specification, p. 19, lines 10-19; p. 21, lines 20-23; p. 35, line 26, through p. 39, line 27; p. 45, line 13 through p. 46, line 11.

For at least the reasons above, the specification provides sufficient guidance to enable the skilled artisan to practice the claimed methods without undue experimentation. *See Wands*, 8 U.S.P.Q.2d at 1404. Accordingly, the rejection is improper and should be withdrawn.

II

Claims 33-36

Claims 33-53 are rejected under 35 U.S.C. § 112, first paragraph, as allegedly directed to subject matter not enabled by the specification. Applicants traverse the rejection as it applies to amended claims 33-36; the rejection is moot with respect to claims 37-53 by the cancellation of the claims without prejudice or disclaimer.

(A) Nature of the invention:

The present claims are directed to a method of treating a disorder characterized by convulsive seizures in an individual. The specification discloses that the allowed compounds demonstrate statistically significant anticonvulsant activity at 30 and 45 mg/kg doses in a mouse Maximal Electroshock (MES) test. *See, e.g.*, Specification, p. 47, line 11, through p. 49, line 16. The MES test is one of several standard models of tonic-clonic seizure activity. *See, e.g.*, Specification, p. 47, lines 13-14; Giardina, "Models of epilepsy: electroshock and chemical induced convulsions in the mouse," *Curr. Protocols Pharm.* 5.22.1-22 (2000) ("Giardina"; attached hereto as **EXHIBIT 1**). Drugs that block seizures and convulsions in the MES test are considered candidates for treating human clonic and/or tonic generalized seizures common to epilepsy. *See* Giardina, p. 5.22.1, ¶ 2; p. 5.22.5, ¶ under alternate protocol. The skilled artisan thus reasonably expects compounds effective in the MES test to treat a disorder, e.g., epilepsy, characterized by convulsive seizures in an individual.

(B) Breadth of the claims:

As stated above, the claims are directed to a method of treating a disorder characterized by convulsive seizures in an individual. Giving "treating" its broadest reasonable interpretation, the claims encompass methods of alleviating or reducing the incidence of convulsive seizures in an individual, even if the methods do not result in a complete cure of convulsive seizures. *See Cortright*, 49 U.S.P.Q.2d at 1469.

(C) State of the relevant art:

The Office alleges that the state of the art is unpredictable, because of the results disclosed in Gomtsyan *et al.*, *Curr. Pharm. Design* 10: 1093-1103 (2004) (cited by the Office). Gomtsyan teaches that adenosine kinase inhibitors have proven anti-convulsive activity in animal models, among other things. *See* Gomtsyan, Abstract. The Office discounts these results, however, because clinical trials were halted in view of serious side effects of one of the tested compounds. First, the compounds tested in Gomtsyan are not one of the claimed compounds, so Gomtsyan does not provide that undue experiment would be required to use the claimed compounds. Second, as stated above, the artisan would expect compounds with efficacy in the MES test to have some ability to treat convulsive seizures, irrespective of results in clinical trials. In this regard, it is well established that the legal standard for compliance with the enablement requirement is separable from any requirement for efficacy in clinical trials. In short, whether a compound is effective in clinical trials is for the FDA—not the PTO—to determine: "Title 35 does not demand that such human testing occur within the confines of [PTO] proceedings." *Brana*, 34 U.S.P.Q.2d at 1442.

(D) Working example:

The specification provides a working example of a method of treating a disorder characterized by convulsive seizures in an individual, where it discloses that the present compounds significantly reduce convulsions in the MES test. *See, e.g.*, Specification, p. 47, line 11, through p. 49, line 16. Efficacy in an animal model may serve as a working example of efficacy in a method treatment, if the skilled artisan would accept the animal model as reasonably correlating to the condition to be treated. *See, e.g.*, *Brana*, 34 U.S.P.Q.2d at 1441. For the reasons set forth in Part II, 8(A) above, the skilled artisan considers efficacy in the MES test to correlate reasonably with the ability of a compound to

treat convulsions in a therapeutic method of treatment. *See, e.g.*, Giardina, p. 5.22.1, ¶ 2. It follows that the successful *in vitro* testing of the claimed compounds constitutes a working example of the claimed method of treating a disorder characterized by convulsive seizures in an individual.

(E) Additional guidance:

The specification discloses at length pharmaceutical compositions that are useful for the present methods. The disclosure also guides the selection of effective amounts, routes of administration, etc. *See, e.g.*, Specification, p. 19, lines 20-24; p. 25, lines 3-8; p. 28, lines 14-19; p. 35, line 26, through p. 39, line 27; p. 47, line 11, through p. 49, line 16.

For at least the reasons above, the specification provides sufficient guidance to enable the skilled artisan to practice the claimed methods without undue experimentation. *See Wands*, 8 U.S.P.Q.2d at 1404. Accordingly, the rejection is improper and should be withdrawn.

CONCLUSION

In conclusion, this amendment and reply is believed to be a full response to the outstanding Office Action. Should any issues remain outstanding or if there are any questions concerning this paper, or the application in general, the Examiner is invited to telephone the undersigned representative at the Examiner's earliest convenience. If there are any other fees due in connection with the filing of this response, please charge the fees to our Deposit Account No. 50-0573.

Respectfully submitted

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EXHIBIT 1

Giardina, "Models of epilepsy: electroshock and chemical induced convulsions in the mouse," *Curr. Protocols Pharm.* 5.22.1-22 (2000).

Models of Epilepsy: Electroshock and Chemical Induced Convulsions in the Mouse

Epilepsy is a disorder of the central nervous system (CNS) that is characterized by seizures, which are sudden, unprovoked, transitory, and recurrent episodes of abnormal hypersynchronous neuronal discharge. Epileptic seizures are classified into partial seizures (with a focal or local cortical origin and clonic motor movements) and generalized seizures (involving the entire brain from the onset) that are either convulsive (with clonic or tonic-clonic muscle movements) or nonconvulsive (without muscle movements) in nature (McNamara, 1996). Clonic convulsions consist of a prolonged and rapid succession of involuntary muscle contractions, while tonic convulsions are characterized by predominant abduction and extension of the limbs and rigid stretching of the body. The induction of clonic and tonic convulsive seizures that reflect partial and generalized epilepsy is the foundation of the basic animal models of epilepsy.

The antiseizure or anticonvulsive pharmacology of novel test substances can be characterized using variations of two basic test methods in mice: blockade of electroshock-induced convulsive seizures and blockade of chemical-induced convulsive seizures. In the maximal electroshock (MES) test, convulsive seizures are induced by applying a sufficiently strong electric current to the brain to initiate a seizure event that spreads throughout the CNS. Four of the most effective drugs that control partial and generalized tonic-clonic seizures in human epilepsy are phenytoin, carbamazepine, valproic acid, and phenobarbital. These drugs block MES seizures in mice, and thus MES seizures are considered an animal model of human clonic and/or tonic generalized seizures. The most frequently used chemical convulsant is pentylenetetrazol (PTZ). In mice, PTZ is administered subcutaneously at a dose of 85 mg/kg to induce mostly clonic convulsions. PTZ seizures are blocked by ethosuximide, valproic acid, phenobarbital, and diazepam, drugs that are used in controlling generalized absence seizures and/or myoclonic seizures (shocklike contractions of muscles) in epilepsy. PTZ-induced clonic seizures are therefore considered a model of myoclonic/generalized absence epilepsy. Using these basic tests, an investigator can identify and differentiate the anticonvulsant pharmacology of novel compounds. Additional anticonvulsant testing using more elaborate models, such as kindled seizures and genetically epilepsy-prone animals, is needed to more fully characterize the anticonvulsant potential of any test substance before clinical study (see Background Information). This unit presents protocols for detecting the anticonvulsant activity of test substances using the basic MES (see Basic Protocol 1) and PTZ (see Basic Protocol 2 and Alternate Protocol 2) methods in mice. The unit also describes protocols for the threshold MES test (see Alternate Protocol 1), the PTZ infusion test (see Basic Protocol 3), and the administration of bicuculline, picrotoxin, or strychnine (see Basic Protocol 4). In addition, the unit describes how the MES and PTZ methods can be used to detect the proconvulsant activity of test substances, that is, the ability of test substances to predispose the brain to seizures (see Basic Protocols 2 and 3 and Alternate Protocol 1). Although mice are used in all the protocols, rats are often used in anticonvulsant testing.

To conduct these experiments, the investigator must be able to recognize clonic and tonic convulsions in the mouse. One of the easiest ways to demonstrate the different seizures in mice is to administer PTZ to small groups of animals ($N = 4$) at doses of 80, 85, 90, 100, 110, and 120 mg/kg, subcutaneously, and observe the various seizure events. Clonic convulsions in the mouse are defined by episodes of repetitive muscle spasms that persist for at least 5 sec. Tonic extensor convulsions in the mouse are characterized by the rigid extension of the hind limbs that exceeds a 90° angle with the body. Clonic convulsions

Contributed by William J. Giardina
Current Protocols in Pharmacology (2000) 5.22.1-5.22.22

occur at low doses of PTZ (e.g., 85 mg/kg, subcutaneously) and both clonic and tonic convulsion are seen at higher doses (e.g., 125 mg/kg, subcutaneously).

Members of animal care and use committees must be given assurances that animals are treated humanely in seizure experiments. One frequently asked question is whether animals suffer pain during seizures. The answer is that no one knows what the animal feels during a seizure event. As a precaution against unnecessary suffering, animals are euthanized by CO_2 gas inhalation immediately after a failed first attempt to initiate an electroshock seizure and immediately upon completion of an experimental observation period after electrical and chemical-induced seizures.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

BASIC PROTOCOL 1

ANTICONVULSANT DETERMINATION USING ELECTROSHOCK-INDUCED CONVULSIONS IN THE MOUSE

This protocol describes the use of electroshock-induced convulsions to characterize the anticonvulsant pharmacology of test substances. Animals are administered test substances by intraperitoneal injection, and after a suitable period of time for absorption (15 to 30 min) are subjected to an electroshock-induced convulsion. The same protocol can be used with oral or subcutaneous dosing of test substances with appropriate adjustments in time for absorption (30 to 60 min). A typical maximal electroshock-induced convulsion consists of a brief tonic flexor convulsion (flexion) and a prolonged tonic extensor convulsion (extension), particularly of the hind limbs. These tonic seizures are followed by a terminal clonic seizure. Blockade of the hind-limb tonic extensor component of the convulsion indicates an anticonvulsant effect. Carbamazepine (10 mg/kg, intraperitoneal; 30 mg/kg, oral) is used as a positive reference anticonvulsant compound in this test.

Materials

CD1 or NMRI male mice, 25 to 35 g, group housed on a 24-hr diurnal cycle (lights on at 0700 and off at 1900), fed standard rodent diet
Vehicle: distilled water, saline, or 0.2% (w/v) hydroxypropylmethylcellulose (HPMC; see step 2)
Reference compound: e.g., carbamazepine (Sigma) in 0.2% (w.v) HPMC
Test substance
Topical anesthetic: 0.5% tetracaine·HCl (J.A. Webster)
Electrode gel (Grass Telefactor, Astro-Med)

Transparent plastic cages, 59 cm × 39 cm × 14 cm, with bedding
Electroconvulsive shock apparatus with corneal stimulating electrodes (Stoelting Co.)
CO_2 gas chamber, made in-house using a Nalgene Multipurpose Jar (13-cm diameter, 19-cm high) with a hole in the lid for a 0.5-in gas tube from a CO_2 gas cylinder equipped with a regulator valve.

1. Bring mice, 10 for the vehicle group, the reference compound group (optional), and each dose group, to the laboratory. Allow mice to acclimate to the test room for 30 min before use.

2. Prepare the test substance for dosing in a volume of 10 ml/kg of body weight in an appropriate vehicle (water, saline, or suspending vehicle) for the first dosing group.

Soluble compounds: Dissolve soluble test substances and convulsants in sterile water for injection or sterile physiological saline for intraperitoneal, subcutaneous or intravenous administration. Dissolve test substances in distilled water for oral administration.

Insoluble compounds: Disperse insoluble test substances in 0.2% (w/v) hydroxypropylmethylcellulose (HPMC) (Sigma-Aldrich) in distilled water for intraperitoneal or oral administration. If the solubilty of any test substance or reference compound is uncertain, disperse it in HPMC and administer it by the intraperitoneal or oral route.

Solution for Injection: Weigh an amount of test substance in mg equal to the mg dose of test substance to be administered and dissolve or disperse in 10 ml of vehicle (e.g., for 10 mg/kg dose of carbamazepine, disperse 10 mg of carbamazepine in 10 ml of 0.2% (w/v) hydroxypropylmethylcellulose in distilled water). To administer the test substance at 10 ml/kg body weight, administer 0.1 ml of the stock solution for each 0.01 kg of body weight (e.g., a 0.03 kg mouse receives 0.3 ml of stock solution).

3. Select 10 mice, record their weight, and place a unique identifying mark on each animal with a permanent-ink black marker. Place the group in a transparent plastic holding cage to await dosing.

4. Administer the test substance, reference compound, or vehicle to each mouse. Stagger the administration (1- to 5-min intervals) to maintain the same time between dosing and testing for each animal.

5. Place mice together in the transparent plastic holding cage as they are dosed.

6. Set an electroshock apparatus to deliver a 50-mA stimulus, with a 0.4-sec duration, a pulse width of 0.5 msec and a frequency of 60 pulses/sec.

   The optimal current (mA), duration (sec), pulse width (msec), and frequency (Hz) settings of the electroshock must be determined empirically, as they can vary with the weight and strain of the mice. It is best to establish these parameters in a pilot study using vehicle-treated mice. It is also important to adjust the spacing of the corneal electrodes (see step 7) in the pilot study to suit the size of the mice; it takes practice to deliver the electroshock stimulus reliably. Electroshock stimulation can also be delivered via auricular (earlobe) clip electrodes.

Administer electrical stimulation

7. Coat corneal electrodes with electrode gel to insure good contact with the corneas.

8. Place a drop of topical anesthetic on the eyes of the mice.

9. Begin testing mice when the post-treatment time has elapsed. Grasp a mouse firmly by the nape of the neck, lift the mouse between the corneal electrodes so that its corneas contact the electrodes, and initiate the electrical stimulation.

10. Immediately release the mouse into a separate observation cage to observe the course of the seizure.

11. Record the presence or absence of hindlimb tonic extensor convulsion (extension of the hind legs >90° from plane of body) and death, as shown in Table 5.22.1.

    Observe the mouse for 30 sec to evaluate if a seizure has been induced. If a seizure is induced within 30 sec, observe the mouse for 30 sec following a tonic hindlimb convulsion, and record whether the animals dies during this period.

12. Euthanize in a CO_2 chamber any mouse that does not convulse within 30 sec, or that survives a seizure.

Table 5.22.1 Effects of Carbamazepine and Valproic Acid on Maximal Electroshock-Induced Seizure in Mice[a]

Treatment	Mouse number	Seizure event: Tonic convulsion	Seizure event: Death
Vehicle 10 ml/kg, i.p. 15 min before MES			
	1	+	+
	2	+	+
	3	+	+
	4	+	+
	5	+	+
	6	+	+
	7	+	+
	8	+	+
	9	+	+
	10	+	+
Number protected		0	0
Carbamazepine 10 mg/kg, i.p. 15 min before MES			
	11	Protected	Protected
	12	+	Protected
	13	Protected	Protected
	14	Protected	Protected
	15	+	Protected
	16	Protected	Protected
	17	+	Protected
	18	Protected	Protected
	19	+	Protected
	20	+	Protected
Number protected		5	10
Fisher's exact test		$p < 0.05$	$p < 0.001$
P value		0.0325	0.0001
Valproic acid 130 mg/kg, i.p. 15 min before MES			
	21	+	+
	22	Protected	Protected
	23	+	+
	24	+	+
	25	+	+
	26	Protected	Protected
	27	Protected	Protected
	28	+	Protected
	29	Protected	Protected
	30	+	+
Number protected		4	5
Fisher's exact test		NS	$p<0.05$
P value		0.0867	0.0325

continued

Table 5.22.1 Effects of Carbamazepine and Valproic Acid on Maximal Electroshock-Induced Seizure in Mice[a], continued

Treatment	Mouse number	Seizure event	
		Tonic convulsion	Death
Valproic acid 260 mg/kg, i.p. 15 min before MES			
	31	+	+
	32	+	Protected
	33	Protected	Protected
	34	Protected	Protected
	35	Protected	Protected
	36	Protected	Protected
	37	Protected	Protected
	38	Protected	Protected
	39	Protected	Protected
	40	Protected	Protected
Number protected		8	9
Fisher's exact test		$p < 0.001$	$p < 0.001$
P value		0.0007	0.0001

[a]NS, not significant; i.p., intraperitoneal; MES, maximal electroshock.

Repeat electrical stimulation for all of the mice

13. Repeat steps 7 to 12 for all of the mice in the group in the order they were injected.

14. Repeat entire protocol for each dosing group of 10 mice and the vehicle and reference compound groups.

Calculate results

15. Express the data as the number of mice protected from hindlimb tonic extensor convulsions and death in each dose group (Table 5.22.1).

    Fisher's exact test is used to determine statistical significance between vehicle and individual dose groups for seizures and deaths.

ANTICONVULSANT DETERMINATION BY THE METHOD OF LIMITS USING ELECTROSHOCK-INDUCED CONVULSIONS IN THE MOUSE

ALTERNATE PROTOCOL 1

This protocol describes a variant procedure in which electroshock-induced convulsions are used to characterize the anticonvulsant pharmacology of test substances, but the shock intensity is adjusted from mouse to mouse to determine the seizure threshold (the method of limits). This method can also be used to characterize the proconvulsant properties of test substances. Animals are administered test substances, usually by intraperitoneal injection, and after a suitable period of time for absorption (generally 15 to 30 min) are subjected to an electroshock-induced convulsion. The same protocol can be used with oral or subcutaneous dosing of test substances with appropriate adjustments in time for absorption (30 to 60 min). The first animal in a dose group of 15 is administered electroshock at 30 mA. If there are no tonic convulsions, a second animal is administered electroshock at 40 mA. This procedure is repeated with 10 mA increments until an animal has a tonic convulsion. After an animal has a tonic convulsion, the intensity of the electroshock is decreased by 5 mA for the next animal. The electroshock intensity is then

increased or decreased by 5 mA from animal to animal, depending on whether the preceding animal convulsed. The maximum intensity used is 95 mA. An increase in seizure threshold above that observed in vehicle-treated animals indicates an anticonvulsant effect of the test substances, whereas a decrease in seizure threshold relative to vehicle controls indicates a proconvulsant effect. Diazepam (2 mg/kg, intraperitoneal; 8 mg/kg, oral) and RO 15-4513 (16 mg/kg, intraperitoneal; 64 mg/kg, oral) are often used as anti- and proconvulsant reference compounds, respectively, in this test.

Additional Materials (also see Basic Protocol 1)

Reference compound(s): e.g., diazepam (Sigma) and RO 15-4513 (Sigma) in 0.2% (w/v) HPMC

NOTE: Do not deliver currents stronger than 95 mA during this procedure.

1. Bring mice, 15 for the vehicle group, the reference compound group(s), and each test substance dose group, to the laboratory. Allow mice to acclimate to the test room for at least 30 min before use.

2. Prepare the test substance (see Basic Protocol 1, step 2).

3. Select 15 mice, record their weight, place a unique identifying mark on each animal with a permanent-ink black marker, and place the group in a transparent plastic holding cage to await dosing.

4. Administer the test substance, vehicle, or (optional) reference compound (see Basic Protocol 1, steps 4 and 5).

5. Set the electroshock apparatus to deliver initially a 30-mA stimulus, with a 0.4-sec duration, a pulse width of 0.5 msec, and a frequency of 60 pulses/sec.

   It is best to establish the electroshock parameters in a pilot study using vehicle-treated mice, as these can vary by the weight and strain of mouse. It is also important to adjust the spacing of the corneal electrodes in the pilot study to suit the size of the mice. It takes practice to deliver electroshock stimulus reliably.

Begin limits testing

6. Administer electrical stimulation when the posttreatment time has elapsed (see Basic Protocol 1, steps 7 to 10).

   Administer electrical stimulation at 30 mA to the first animal and increase or decrease the current strength thereafter as indicated below.

7. Record the current strength and the presence or absence of hind limb tonic convulsion (extension of the hind legs >90° from plane of body) and death for each animal as shown in Table 5.22.2. (See Basic Protocol 1, step 11).

8. Euthanize in a CO_2 chamber any mouse that does not convulse within 30 sec, or that survives a seizure.

Change current strength according to the presence or absence of a convulsion

9. If there is no tonic convulsion, administer electroshock at 40 mA to the second animal. Repeat the procedure in steps 6 to 8 with the subsequent animals, with 10-mA increments in current until an animal has a tonic convulsion.

10. Decrease the current by 5 mA, and repeat steps 6 to 8 for the next animal.

11. Repeat steps 6 to 8 with the rest of the animals, increasing the current by 5 mA if the previous animal did not convulse and decreasing it by 5 mA if the previous animal did convulse.

Table 5.22.2 Effects of the Anticonvulsant Diazepam and the Proconvulsant RO-15-4513 on MES Threshold in the Mouse[a]

Treatment	Mouse number	MES intensity in mA	Tonic convulsion	Death
Vehicle 10 ml/kg, p.o. 60 min before MES				
	1	30	No	No
	2	40	Yes	Yes
	3	35	No	No
	4	40	Yes	No
	5	35	Yes	No
	6	30	No	No
	7	35	No	No
	8	40	Yes	No
	9	35	No	No
	10	40	Yes	No
	11	35	Yes	No
	12	30	No	No
	13	35	Yes	Yes
	14	30	No	No
	15	35	Yes	No
Total				2
Mean (±SEM)		35.0 (1.0)		
Diazepam 8 mg/kg, p.o. 60 min before MES				
	16	30	No	No
	17	40	No	No
	18	50	No	No
	19	60	No	No
	20	70	No	No
	21	80	No	No
	22	90	No	No
	23	95	No	No
	24	95	No	No
	25	95	Yes	No
	26	90	Yes	No
	27	85	No	No
	28	90	No	No
	29	95	Yes	No
	30	90	No	No
Total				0
Fisher's exact test				NS
Mean (±SEM)		77.0 (5.6)		
Student's t value		7.332		
Probability		$p < 0.001$		

continued

Table 5.22.2 Effects of the Anticonvulsant Diazepam and the Proconvulsant RO-15-4513 on MES Threshold in the Mouse[a], continued

Treatment	Mouse number	MES intensity in mA	Tonic convulsion	Death
RO 15-4513 64 mg/kg, p.o. 60 min before MES				
	31	30	No	No
	32	40	Yes	No
	33	35	Yes	No
	34	30	Yes	No
	35	25	No	No
	36	30	Yes	No
	37	25	No	No
	38	30	No	No
	39	35	Yes	No
	40	30	No	No
	41	35	Yes	No
	42	30	Yes	No
	43	25	No	No
	44	30	Yes	No
	45	25	No	No
Total				0
Fisher's exact test				NS
Mean (±SEM.)		30.3 (1.1)		
Student's *t* value		3.108		
Probability		p <0.01		

[a]NS, not significant, p.o., orally administered; SEM, standard error of the mean.

Repeat the protocol for the rest of the groups

12. Repeat steps 5 to 11 for each dosing group of 15 mice, the vehicle group, and reference compound group(s).

Calculate results

13. Calculate the mean and the standard error of the mean (SEM) of the current intensity (mA) data recorded (see Table 5.22.2).

A Student's t-test or one-way analysis of variance with post hoc test is used to detect significant differences in current intensity among the vehicle and dose groups. Fisher's exact test is used to determine whether there is a statistically significant difference in the number of deaths between vehicle and individual dose groups.

BASIC PROTOCOL 2

PROCONVULSANT/ANTICONVULSANT DETERMINATION USING PENTYLENETETRAZOL (120 mg/kg, SUBCUTANEOUS)–INDUCED CLONIC AND TONIC CONVULSIONS IN THE MOUSE

This protocol describes the use of convulsions induced by subcutaneous injection of pentylenetetrazol (PTZ) to characterize the anticonvulsant/proconvulsant pharmacology of test compounds. Animals are administered test substances by intraperitoneal injection, and after a suitable period of time for absorption (15 to 30 min), are then given PTZ. The same protocol can be used with oral or subcutaneous dosing of test substances, with

appropriate adjustments in time for absorption (30 to 60 min). The blockade of episodes of clonic and tonic convulsion indicates an anticonvulsant effect, whereas a decrease in seizure latency indicates a proconvulsant effect of the test substance. A dose of 120 mg/kg, subcutaneous, of PTZ produces clonic and tonic convulsions and death in most mice. Diazepam (2 mg/kg, intraperitoneal; 8 mg/kg, oral) and RO-15-4513 (16 mg/kg, intraperitoneal; 64 mg/kg, oral) are used as anticonvulsant and proconvulsant reference compounds, respectively, in this test.

Materials

CD1 or NMRI male mice (25 to 35 g), group housed on a 24-hr diurnal cycle (lights on at 0700 and off at 1900), fed standard rodent diet

Vehicle: distilled water, saline, or 0.2% (w/v) hydroxypropylmethylcellulose (see step 2)

Reference compounds: e.g., diazepam (Sigma) and RO-15-4513 (Sigma) in 0.2% (w/v) HPMC

Pentylenetetrazol (PTZ; Sigma) in saline

Holding cage, transparent plastic, 59 cm × 39 cm × 14 cm, with bedding

Individual observation cages, transparent plastic, 27 cm × 21 cm × 14 cm, with bedding, one for each mouse in a testing group

Timers, one for each observation cage

CO_2 gas chamber

1. Bring mice, 10 for the vehicle group, the reference compound group(s), and each dose group, to the laboratory. Allow mice to acclimate to the test room for at least 30 min before use.

2. Prepare a solution of the test substance for dosing in a volume of 10 ml/kg of body weight in an appropriate vehicle (water, saline, or suspending vehicle) for the first dosing group.

   Soluble compounds: Dissolve soluble test substances and convulsants in sterile water for injection or sterile physiological saline for intraperitoneal, subcutaneous or intravenous administration. Dissolve test substances in distilled water for oral administration.

   Insoluble compounds: Disperse insoluble test substances in 0.2% (w/v) hydroxypropylmethylcellulose (HPMC) (Sigma-Aldrich) in distilled water for intraperitoneal or oral administration. If the solubilty of any test substance or reference compound is uncertain, disperse it in HPMC and administer it by the intraperitoneal or oral route.

   Solution for Injection: Weigh an amount of test substance in mg equal to the mg dose of test substance to be administered and dissolve or disperse in 10 ml of vehicle (e.g., for 10 mg/kg dose of carbamazepine, disperse 10 mg of carbamazepine in 10 ml of 0.2% (w/v) hydroxypropylmethylcellulose in distilled water). To administer the test substance at 10 ml/kg body weight, administer 0.1 ml of the stock solution for each 0.01 kg of body weight (e.g., a 0.03 kg mouse receives 0.3 ml of stock solution).

3. Prepare PTZ solution for dosing at 120 mg/kg, subcutaneous, in a dosing volume of 10 ml/kg body weight.

4. Select 10 mice for the first dosing group and record their weight. Place a unique identifying mark on each animal with a permanent-ink black marker and place the group in a holding cage to await dosing.

5. Administer test substance, vehicle, or reference compound, staggering the administration (1- to 5-min intervals) to maintain the same time interval between test substance dosing and PTZ administration for each animal.

6. Place mice in individual transparent observation cages after dosing.

Table 5.22.3 Effects of Diazepam and RO 15-4513 on Pentylenetetrazol (PTZ) (120 mg/kg)-Induced Seizures in Mice[a]

Treatment	Mouse number	Clonic convulsions		Tonic convulsions		Death	
		Presence	Latency (sec)	Presence	Latency (sec)	Presence	Latency (sec)
Vehicle 10 ml/kg, p.o. 60 min before PTZ							
	1	+	660	–	1800	–	1800
	2	+	120	+	990	+	1050
	3	+	150	–	1800	–	1800
	4	+	150	+	1020	–	1800
	5	+	390	–	1800	–	1800
	6	+	180	+	570	+	600
	7	+	600	+	600	+	690
	8	+	750	+	750	+	810
	9	+	120	+	810	–	1800
	10	+	960	+	960	+	1470
Total		10		7		5	
Mean (±SEM)			408.0 (98.5)		1110.0 (157.9)		1362.0 (163.5)
RO 15-4513 64 mg/kg, p.o. 60 min before PTZ							
	11	+	120	+	390	+	1230
	12	+	90	+	420	+	570
	13	+	120	+	120	+	420
	14	+	90	+	120	+	420
	15	+	90	+	390	+	480
	16	+	90	+	300	+	600
	17	+	120	+	450	–	1800
	18	+	90	+	210	+	300
	19	+	90	+	360	–	1800
	20	+	60	+	390	+	480
Total		10		10		5	
Fisher's exact test		NS		NS		NS	
Percent antagonism		0%		–43%		–60%	
Mean (±SEM)			96.0 (6.0)		315.0 (38.8)		810.0 (183.2)
Student's t value			3.161		4.889		2.248
Probability			$p < 0.01$		$p < 0.001$		$p < 0.05$

continued

Table 5.22.3 Effects of Diazepam and RO 15-4513 on Pentylenetetrazol (PTZ) (120 mg/kg)-Induced Seizures in Mice[a], continued

Treatment	Mouse number	Clonic convulsions		Tonic convulsions		Death	
		Presence	Latency (sec)	Presence	Latency (sec)	Presence	Latency (sec)
Diazepam 8 mg/kg, p.o. 60 min before PTZ							
	21	–	1800	–	1800	–	1800
	22	–	1800	–	1800	–	1800
	23	–	1800	–	1800	–	1800
	24	–	1800	–	1800	–	1800
	25	–	1800	–	1800	–	1800
	26	–	1800	–	1800	–	1800
	27	–	1800	–	1800	–	1800
	28	–	1800	–	1800	–	1800
	29	–	1800	–	1800	–	1800
	30	–	1800	–	1800	–	1800
Total		0		0		0	
Fisher's exact test		$p < 0.001$		$p < 0.01$		$p < 0.05$	
Percent antagonism		100%		100%		100%	
Mean (±SEM)			1800		1800		1800
Student's t value			NC		NC		NC
Probability			NC		NC		NC

[a]NC, not calculated, NS, not significant; p.o., orally administered; SEM, standard error of the mean.

Inject PTZ

7. Begin injecting mice with PTZ when the post-treatment time has elapsed. Grasp a mouse firmly and inject PTZ subcutaneously into the nape of the neck, just caudal to the cranium.

   Continue to inject the remaining mice in the group with PTZ, in the order they were dosed and maintain the same time interval, while observing those that were previously injected (steps 8 to 12). With practice, one investigator can observe 10 mice simultaneously.

8. Return the mouse immediately to the observation cage, and start a timer.
9. Observe each mouse for 30 min after PTZ injection.
10. Record the presence of clonic convulsions that persist for 5 sec, tonic convulsions, and death.
11. Record the presence or absence of a clonic and tonic convulsion and death, and the latency to the clonic and tonic convulsion and death; assign a value of 1800 sec (the maximum observation time) to mice that do not have convulsions.
12. Use mice only once and euthanize in a CO_2 gas chamber at the end of the observation period or immediately after a potentially lethal seizure event.

Repeat for all of the mice

13. Repeat steps 4 to 12 for each dosing group of 10 mice, the vehicle group, and the reference compound group(s).

Calculate the results

14. Express the data as the number of mice having clonic and/or tonic convulsions and the number of deaths in each dose group. Calculate the mean and the standard error of the mean (SEM) of the latency data (Table 5.22.3).

    An indication of proconvulsant and anticonvulsant effect is obtained by calculating the percentage change in the number of seizure events. That is, data are expressed as the percent of change in the number of convulsions and deaths: the percent change is equal to the control score minus the treatment score divided by the control score, times 100. A positive percent value indicates an anticonvulsant effect whereas a negative percent value indicates a proconvulsant effect. Fisher's exact test is used to determine significance differences in the number of events between vehicle and individual dose groups. A Student's t-test or one-way analysis of variance with a post hoc test is used to detect significant differences in latencies between the vehicle and dose groups. Because diazepam blocks all convulsions and deaths and, thus, the latencies for all of these events equals 1800 sec, there is no need to include these data in the inferential statistical analysis, as it is obvious that diazepam has anticonvulsant effects.

ALTERNATE PROTOCOL 2

ANTICONVULSANT DETERMINATION USING PENTYLENETETRAZOL (85 mg/kg, SUBCUTANEOUS)–INDUCED CLONIC CONVULSIONS IN THE MOUSE

This protocol describes the use of convulsions induced by subcutaneous injection of pentylenetetrazol (PTZ) to characterize the anticonvulsant pharmacology of test substances. Test substances are administered to animals by intraperitoneal injection, then, after a suitable period of time for absorption (15 to 30 min), PTZ is administered. The same protocol can be used with oral or subcutaneous dosing of test substances with appropriate adjustments in time for absorption (30 to 60 min). The blockade of episodes of clonic convulsion or an increase in convulsion latency indicates an anticonvulsant effect. A dose of 85 mg/kg (subcutaneous) of PTZ produces clonic convulsions in most mice and is often described as being effective in at least 90% of mice injected; however, some mice will have tonic convulsions. Diazepam (2 mg/kg, intraperitoneal; 8 mg/kg, oral), and valproic acid (300 mg/kg, intraperitoneal; 800 mg/kg, oral) are often used as reference anticonvulsant compounds in this test.

Additional Materials *(also see Basic Protocol 2)*

Reference compounds: e.g., diazepam (Sigma), and valproic acid (Sigma) in 0.2% (w/v) HPMC

1. Bring mice to the laboratory and prepare test substance (see Basic Protocol 2, steps 1 and 2).
2. Prepare PTZ solution in saline for dosing at 85 mg/kg, subcutaneous., in a dosing volume of 10 ml/kg body weight.
3. Perform the anticonvulsant determination (see Basic Protocol 2, steps 4 to 13), but use a dose of 85 mg/kg PTZ instead of 120 mg/kg.
4. Express the data as number of mice having clonic convulsions in each dose group and calculate the mean and the standard error of the mean (SEM) of the latency data (Table 5.22.4).

    Fisher's exact test is used to determine statistical significance in the number of events between vehicle and individual dose groups. A Student's t-test or one-way analysis of variance with post hoc test (see Table 5.22.4) is used to detect significant differences among latencies in the vehicle and dose groups. Because valproic acid blocks all clonic convulsions, and, thus, all the latencies equal 1800 sec, there is no need to include this group in the inferential statistical analysis, as it is obvious that valproic acid has anticonvulsant effects.

Table 5.22.4 Effects of Valproic Acid on Pentylenetetrazole (PTZ) (85 mg/kg)-Induced Seizures in Mice[a]

Treatment	Mouse number	Clonic convulsion	
		Presence	Latency (sec)
Vehicle 10 ml/kg, i.p. 15 min before PTZ			
	1	+	267
	2	+	189
	3	+	242
	4	–	1800
	5	+	199
	6	+	749
	7	+	594
	8	+	221
	9	+	204
	10	+	322
Total		9	
Mean (±SEM)			479 (159)
Valproic acid 30 mg/kg, i.p. 15 min before PTZ			
	11	+	199
	12	+	247
	13	+	91
	14	+	149
	15	+	207
	16	+	152
	17	+	107
	18	+	526
	19	+	344
	20	+	119
Total		10	
Fisher's exact test		1.000	
Probability		NS	
Mean (±SEM)			214 (42)
Student's t value			1.613
Probability			NS
Valproic acid 100 mg/kg, i.p. 15 min before PTZ			
	21	+	259
	22	+	681
	23	+	187
	24	+	338
	25	–	1800
	26	+	424
	27	+	627
	28	+	497
	29	+	1192
	30	+	307
Total		9	
Fisher's exact test		1.5263	
Probability		NS	
Mean (±SEM)			631 (159)
Student's t value			0.6795
Probability			NS

continued

Table 5.22.4 Effects of Valproic Acid on Pentylenetetrazole (PTZ) (85 mg/kg)-Induced Seizures in Mice[a], continued

Treatment	Mouse number	Clonic convulsion	
		Presence	Latency (sec)
Valproic acid 200 mg/kg, i.p. 15 min before PTZ			
	31	–	1800
	32	+	465
	33	+	679
	34	+	366
	35	–	1800
	36	+	317
	37	+	275
	38	+	1283
	39	–	1800
	40	–	1800
Total		6	
Fisher's exact test		0.0001	
Probability		p <0.001	
Mean (±SEM)			1058 (221)
Student's t value			2.133
Probability			p <0.05
Valproic acid 300 mg/kg, i.p. 15 min before PTZ			
	41	–	1800
	42	–	1800
	43	–	1800
	44	–	1800
	45	–	1800
	46	–	1800
	47	–	1800
	48	–	1800
	49	–	1800
	50	–	1800
Total		0	
Fisher's exact test		0.0001	
Probability		p <0.001	
Mean (±SEM)			1800
Student's t value			NC
Probability			NC

[a]NC, not calculated, NS, not significant; i.p. intraperitoneal; SEM, standard error of the mean.

BASIC PROTOCOL 3

ANTICONVULSANT DETERMINATION USING A TIMED INTRAVENOUS INFUSION OF PENTYLENETETRAZOL TO INDUCE CONVULSIONS IN THE MOUSE

This protocol describes the use of convulsions induced by the timed intravenous infusion of a 0.5% solution of pentylenetetrazol (PTZ) to characterize the potential proconvulsant and anticonvulsant pharmacology of test compounds. Animals are administered test substances by intraperitoneal injection, then, after a suitable period of time for absorption (15 to 30 min), are infused with PTZ. The same protocol can be used with oral or subcutaneous dosing of test substances with appropriate adjustments in time for absorption (30 to 60 min). The volume of PTZ infused at the onset of three distinct sequential seizure events—(1) first twitch, (2) pseudoclonus, and (3) tonic extensor convulsion—is

recorded. First twitch is a sharp twitch of the entire body. Pseudoclonus is characterized by short-lived clonic movements and squeaking. Optimal results are obtained when the mice in all of the dosing groups are nearly of equal weight. Proconvulsant compounds decrease the volume of PTZ required to initiate each seizure event and anticonvulsant compounds increase it. In contrast to the methods using PTZ described in the Basic Protocol 2 and Alternate Protocol 2, all mice in this protocol have seizures because the PTZ is infused until seizures occur. There is no fixed dose of PTZ and no maximum observation time. All treatment groups are thus included in the statistical analysis of changes in seizure threshold from vehicle control. The disadvantage of this protocol is that only one mouse is tested at a time and the experimenter must maintain an intravenous tail infusion throughout the observation period. Diazepam (2 mg/kg, intraperitoneal; 8 mg/kg, oral) and RO-15-4513 (16 mg/kg, intraperitoneal; 64 mg/kg, oral) are used as anticonvulsant and proconvulsant reference compounds, respectively, in this test.

Materials

Male mice (25 to 35 g), group housed on a 24-hr diurnal cycle (lights on at 0700 and off at 1900), fed standard rodent diet
Vehicle: distilled water, saline, or 0.2% (w/v) hydroxypropylmethylcellulose (HPMC; see step 2)
Reference compounds: e.g., diazepam (Sigma) and RO-15-4513 (Sigma) in 0.2% (w/v) HPMC
Pentylenetetrazol (PTZ)

Holding cages, transparent plastic, 59 cm × 39 cm × 14 cm, with bedding
Tail access rodent restraint (Stoelting)
27-G needles
Syring pump (Stoelting)
CO_2 gas chamber

1. Bring mice, 10 for vehicle group, the reference compound group(s), and each dose group, to the laboratory. Allow mice to acclimate to the test room for at least 30 min before use.

2. Prepare the test substance for dosing in a volume of 10 ml/kg of body weight in an appropriate vehicle (water, saline, or suspending vehicle) for the first dosing group.

   Soluble compounds: Dissolve soluble test substances and convulsants in sterile water for injection or sterile physiological saline for intraperitoneal, subcutaneous or intravenous administration. Dissolve test substances in distilled water for oral administration.

   Insoluble compounds: Disperse insoluble test substances in 0.2% (w/v) hydroxypropylmethylcellulose (HPMC) (Sigma-Aldrich) in distilled water for intraperitoneal or oral administration. If the solubilty of any test substance or reference compound is uncertain, disperse it in HPMC and administer it by the intraperitoneal or oral route.

   Solution for Injection: Weigh an amount of test substance in mg equal to the mg dose of test substance to be administered and dissolve or disperse in 10 ml of vehicle (e.g., for 10 mg/kg dose of carbamazepine, disperse 10 mg of carbamazepine in 10 ml of 0.2% (w/v) hydroxypropylmethylcellulose in distilled water). To administer the test substance at 10 ml/kg body weight, administer 0.1 ml of the stock solution for each 0.01 kg of body weight (e.g., a 0.03 kg mouse receives 0.3 ml of stock solution).

3. Prepare a 0.5% (w/v) solution of PTZ in saline for intravenous dosing.

4. Select 10 mice of nearly equal weight for the first dosing group and place a unique identifying mark on each animal with a permanent-ink black marker. Place the mice in a holding cage to await dosing.

Table 5.22.5 Effects of Diazepam on Convulsions Induced by the Slow Intravenous Administration of Pentylenetetrazol (PTZ) (0.5% w/v at 0.3 ml/min) in Mice

Treatment	Dose (mg/kg i.p. injected 30 min. before)	First twitch[a]	Pseudoclonus[b]	Persistent convulsion[c]
		Mean onset in total ml of PTZ infused		
Vehicle	—	0.15 ± 0.01	0.20 ± 0.01	0.50 ± 0.04
Diazepam	1.0	0.26 ± 0.01	0.31 ± 0.03	0.59 ± 0.03
	3.0	0.31 ± 0.01	0.36 ± 0.01	0.82 ± 0.03
	10.0	0.57 ± 0.02	0.64 ± 0.02	1.40 ± 0.09

[a]Sharp twitch of entire body.
[b]Short-lived clonic movements and squeaking.
[c]Tonic extensor convulsion.

5. Administer the test substance, reference compound(s) (optional), or vehicle, staggering their administration (10-min intervals) to maintain the same time between dosing and the infusion of PTZ for each animal in the group.

6. Place the animals in the holding cage after dosing.

Infuse with PTZ

7. When the post-treatment time has elapsed, begin infusing the animals with PTZ in the order they were injected. Place a mouse in a mouse restraint, firmly grasp the tail, insert a 27-G needle into the tail vein, and start an infusion pump to deliver PTZ at 0.3 ml/min, and immediately start a laboratory timer.

8. Record the latency and volume of PTZ infused upon observing (1) the first sharp twitch, (2) short-lived clonic movements, and (3) persistent tonic convulsions.

9. Repeat for all of the mice in the order they were injected.

10. Use mice only once and euthanize in a CO_2 gas chamber at the end of the observation period or immediately after a potentially lethal seizure event.

Repeat for all of the mice

11. Repeat steps 4 to 10 for each dosing group of 10 mice, and for the vehicle group and reference compound group(s).

Calculate results

12. Calculate the mean and the standard error of the mean (SEM) of the volume of PTZ solution infused to the start of each seizure event, or calculate the mean and the standard error of the mean (SEM) of the latency data.

A Student's t-test or one-way analysis of variance with post hoc test is used to detect significant differences between the vehicle and dose groups for each seizure event. Threshold changes for each seizure event are expressed as percent change from control: the mean volume of PTZ infused in a dose group divided by the mean volume of PTZ in the vehicle control group, times 100. Values >100 indicate an increase in threshold whereas values <100 indicate a decrease in seizure threshold. Table 5.22.5 shows the tabulated results of a PTZ infusion study.

ANTICONVULSANT DETERMINATION USING BICUCULLINE, PICROTOXIN, OR STRYCHNINE-INDUCED CONVULSIONS IN THE MOUSE

BASIC PROTOCOL 4

This protocol describes the use convulsions induced by subcutaneous injection of either bicuculline (BIC), picrotoxin (PIC), or strychnine (STR) to characterize the anticonvulsant pharmacology of test substances. Animals are administered test substances by intraperitoneal injection, then, after a suitable period of time for absorption (15 to 30 min), are administered BIC, PIC, or STR. The same protocol can be used with oral or subcutaneous dosing of test substances with appropriate adjustments in time for absorption. For BIC and PIC, blockade of episodes of clonic convulsion indicates an anticonvulsant effect, while for STR, blockade of hindlimb tonic extensor convulsion indicates an anticonvulsant effect. A dose of 3.5 mg/kg, subcutaneous, of BIC or STR produces clonic convulsions in most mice, while 3.2 mg/kg subcutaneous PIC produces hindlimb tonic extensor convulsion. Diazepam (2 mg/kg, subcutaneous; 8 mg/kg, oral) is often used as a reference anticonvulsant compound in this test.

Materials *(also see Basic Protocol 2)*

Bicuculline (BIC) *or* picrotoxin (PIC) *or* strychnine (STR) (all available from Sigma)
Reference compound: e.g., diazepam (Sigma) in 0.2% (w/v) HPMC (see step 2)

Individual observation cages, transparent plastic, 27 cm × 21 cm × 14 cm, with bedding, one for each mouse in a testing group
Timers, one for each observation cage

1. Bring mice, 10 for the vehicle, reference compound, and each dose group, to the laboratory. Allow mice to acclimate to the test room for at least 30 min before use.

2. Prepare a solution of the test substance for dosing in a volume of 10 ml/kg of body weight in an appropriate vehicle (water, saline, or suspending vehicle) for the first dosing group.

   Soluble compounds: Dissolve soluble test substances and convulsants in sterile water for injection or sterile physiological saline for intraperitoneal, subcutaneous or intravenous administration. Dissolve test substances in distilled water for oral administration.

   Insoluble compounds: Disperse insoluble test substances in 0.2% (w/v) hydroxypropylmethylcellulose (HPMC) (Sigma-Aldrich) in distilled water for intraperitoneal or oral administration. If the solubilty of any test substance or reference compound is uncertain, disperse it in HPMC and administer it by the intraperitoneal or oral route.

   Solution for Injection: Weigh an amount of test substance in mg equal to the mg dose of test substance to be administered and dissolve or disperse in 10 ml of vehicle (e.g., for 10 mg/kg dose of carbamazepine, disperse 10 mg of carbamazepine in 10 ml of 0.2% (w/v) hydroxypropylmethylcellulose in distilled water). To administer the test substance at 10 ml/kg body weight, administer 0.1 ml of the stock solution for each 0.01 kg of body weight (e.g., a 0.03 kg mouse receives 0.3 ml of stock solution).

3. Prepare a BIC or STR solution in saline for dosing at 3.5 mg/kg or PIC at 3.2 mb/kg, subcutaneously, in a dosing volume of 10 ml/kg of body weight.

4. Select 10 mice, record their weight, and place a unique identifying mark on each animal with a permanent-ink black marker. Place mice in a holding cage to await dosing.

5. Administer the test substance, reference compound, or vehicle (1- to 5-min intervals) to maintain the same time between dosing and the injection of BIC, PIC, or STR for each animal.

6. Place mice in individual transparent observation cages after dosing.

Inject BIC, PIC, or STR

7. When the post-treatment time has elapsed, begin injecting mice with BIC, PIC, or STR solution. Grasp a mouse firmly and inject the solution subcutaneously into the nape of the neck, just caudal to the cranium.

   Continue injecting the mice, in the same order that they were dosed and maintaining the same time interval, while observing the previously injected mice (steps 8 to 11).

8. Return the mouse immediately to the observation cage and start a timer.

9. Observe the mice for 15 min after BIC or PIC injection, or for 30 min after STR injection.

10a. *For BIC- or PIC-injected animals:* Record the presence or absence of a clonic convulsion and the latency to clonic convulsion; assign a value of 1800 sec (the maximum observation time) to mice not having convulsions.

10b. *For STR-injected animals:* Record the presence or absence of a tonic convulsion and the latency to tonic convulsion; assign a value of 1800 sec (the maximum observation time) to mice not having convulsions.

11. Use mice only once and euthanize in a CO_2 gas chamber at the end of the observation period or immediately after a potentially lethal seizure event.

Repeat for all of the mice

12. Repeat steps 4 to 11 for each dosing group of 10 mice and for each vehicle and reference compound group.

Calculate results

13a. *For BIC- or PIC-injected animals:* Express the data as number of mice having clonic convulsions in each dose group and calculate the mean and the standard error of the mean (SEM) of the latency data.

13b. *For STR-injected animals:* Express the data as number of mice having tonic convulsions in each dose group and calculate the mean and the standard error of the mean (SEM) of the latency data.

   Fisher's exact test is used to determine statistical significance in the number of events between vehicle and individual dose groups. A Student's t-test or one-way analysis of variance with post hoc test is used to detect significant differences between latencies in the vehicle and dose groups.

COMMENTARY

Background Information

Convulsive seizures driven by electroshock or chemicals are clearly not epilepsy. Yet, with few exceptions, all anti-epilepsy drugs demonstrate anticonvulsive pharmacology in the MES or PTZ tests, or in both, as shown in Table 5.22.6. The MES and low-dose PTZ (85 mg/kg) tests are primary screening tests. They identify compounds that prevent seizure spread and raise seizure threshold, respectively, because the intensity of the stimulus in each test is set to produce either tonic extension of the hindlimbs, as in the MES test, or clonic convulsion, as in the PTZ (85 mg/kg) test. Compounds that block seizure spread also block hind-limb extension in the MES tests, whereas compounds that block clonic convulsion in the PTZ (85 mg/kg) raise the seizure threshold.

Thousands of compounds have been screened for potential anticonvulsive activity using these tests (Swinyard et al., 1989). No single animal test is sufficient to characterize the anticonvulsive potential of a novel test substance, but the combined results of the MES,

Table 5.22.6 Anticonvulsant Effect of Standard Antiepileptic Drugs Against Different Types of Seizures in Animal Models and in Human Epilepsy[a]

Compound	Anticonvulsant activity in experimental models			Efficacy in epilepsy			
	MES (tonic seizures)	PTZ test (clonic seizures)	Amygdala kindling (focal seizures)	Partial seizures	Generalized seizures		
					Tonic-clonic	Absence	Myoclonic
Valproate	+	+	+	+	+	+	+
Carbamazepine	+	NE	+	+	+	NE	NE
Phenytoin	+	NE	+	+	+	NE	NE
Phenobarbital	+	+	+	+	+	NE	+
Primidone	+	+	+	+	+	NE	+
Benzodiazepines	+	+	+	+	+	+	+
Ethosuximide	NE	+	NE	NE	NE	+	±

[a]Effect is indicated by: +, effective; ±, inconsistent data; NE, not effective. MES, maximal electroshock seizure; PTZ, pentylenetetrazol. Adapted from Loscher (1999).

PTZ, and other seizure tests give the investigator a clear picture of the potential clinical efficacy of a novel test substance (White, 1999).

Besides the MES and PTZ tests, investigators use many other seizure models to complete their characterization of the anticonvulsant properties of test substances. For example, they may use pilocarpine, kainic acid, and methyl-6,7-dimethyoxy-4-ethyl-B-carboline-3-carb oxylate (DMCM)-induced seizures, chemical-induced models of status epilepticus (Walton et al., 1996), genetically epilepsy-prone mice and rats (Buchhalter, 1993; Hosford and Wang, 1997; Sarkisian et al., 1999), photomyoclonic epilepsy in baboons (Buchhalter, 1993), direct cortical ramp stimulation (Krupp and Loscher, 1998), and kindled seizures (McNamara, 1986). Of these, kindled seizures are most often used in conjunction with the MES and PTZ tests as shown in Table 5.22.6. Kindled seizures develop progressively following repeated, low-intensity, localized brain electrical stimulation. Fine stimulating electrodes are permanently implanted in the amygdala or hippocampal brain structures of the rat. A low-intensity current to either area initially induces only a focal after-discharge, but the same current eventually elicits a fully generalized seizure after repeated daily stimulation. During the process of kindling, rats initially develop focal seizures exhibited by head nodding and forelimb clonus, and as kindled seizures becomes more generalized the animals rear and fall when stimulated. The kindling model is considered the best model of focal seizures and complex focal seizures (Loscher, 1999). Test substances are evaluated for their ability to prevent both the development of kindling and to block the expression of fully kindled seizures. All the standard anticonvulsants, with the exception of ethosuximide, block kindled seizures (Table 5.22.6). Mice can also be kindled using repeated, low-intensity, electroshock stimulation, and repeated, low-dose PTZ administration (Matagne and Klitgaard, 1998; De Sarro et al., 1999), although the latter does not produce very consistent kindling effects across different laboratories.

The characterization of the anti-epilepsy potential of a test substance requires the calculation of a protective index (PI), which is the ratio of a CNS side effect to the anticonvulsant efficacy of the test substance. First, an anticonvulsant effective dose 50 (ED_{50}) value—that is, the dose at which 50% of mice are protected from seizures—is determined by testing doses in the range from 0% to 100% seizure protection for both the MES and PTZ tests. The larger the group size per dose of test substance, and the more doses included in the ED_{50} determination, the more reliable the ED_{50} value. ED_{50} determination may not be possible for every test substance, as some will be totally inactive in the MES or PTZ test and others will not produce 100% protection in one test or the other. The ED_{50} value is calculated using linear regression. Next, a toxic dose 50 (TD_{50}) value for motor incoordination is determined in the same manner. A quantitative measure of motor incoordination is obtained by determining whether an animal can remain on a rotating rod (also known as a rotarod apparatus) after a dose of

compound. The TD_{50} value is the dose at which 50% of the animals fail the rotarod test. The PI ratio is the TD_{50} value for rotarod failure divided by the ED_{50} value for seizure protection. A large PI value, for example 10, indicates a potential for a clinically meaningful separation between the sedating or depressant CNS side effects commonly seen in this class of compounds, and the anti-epilepsy activity. The PI can also be determined using other tests of CNS depression, such as locomotor activity. The duration of action and tolerance are also tested as part of the general profiling of anticonvulsive compounds, and are performed using an ED_{50} or ED_{90} dose in either the MES or PTZ test.

In the evaluation of anticonvulsant potential, BIC, PIC, and STR tests are often used to differentiate and characterize the anticonvulsive properties of test substances by comparison with the anticonvulsive profiles of the five standard reference compounds (Swinyard et al., 1989). Valproic acid protects against BIC, PIC, and STR seizures. Ethosuximide and benzodiazepine block BIC and PIC, but have only weak or no protection against STR. In contrast, carbamazepine antagonizes PIC and STR seizures, but has weak protection against BIC, and phenytoin fails to block BIC or PIC and has only weak protection against STR.

An evaluation of the potential pro- and anticonvulsant pharmacology of a test substance is often included in the CNS safety pharmacology profiling study of compounds intended for clinical study. The MES threshold test (see Alternate Protocol 1), the high-dose PTZ (see Basic Protocol 2), and the PTZ infusion tests (see Basic Protocol 3) are used in this capacity. The test substance is administered acutely at four log unit doses to generate dose-response data. The largest dose in the range is generally the maximally tolerated dose on acute injection for behavioral pharmacology studies, as determined in a preliminary observation test for clinical signs of toxicity. In such studies, a proconvulsant, RO-15-4513, and an anticonvulsant, diazepam, are used as active reference substances. The results of these safety studies are most important when they reveal a statistically significant, dose-related decrease in seizure threshold, since clinical investigators need to be aware of the potential proconvulsant pharmacology of test substances at supertherapeutic doses during the Phase I rising dose studies in humans.

Critical Parameters

It is important to perform anticonvulsant experiments smoothly and patiently at the same time period every day, as seizure thresholds may change in agitated or excited mice. The limiting step in seizure studies is often how well an investigator can observe individual mice in separate cages for the presence or absence of seizures, note the type of seizure, and record the latency to first seizure, if that is also part of the experiment, while still meeting an injection schedule. It is generally more efficient for two investigators to work as a team than for a single person to both make injections and record seizure events.

Electroshock stimulus

The most critical parameter in the maximal electroshock seizure test is the intensity of the electroshock stimulus. Most electroshock stimulators allow the investigator to adjust the current (mA), duration (sec), pulse width (msec), and frequency (Hz) of the electroshock. The electrical stimulation parameters described in Basic Protocol 1 should be adequate and a good starting point for the standard MES test. Technique in the MES test is also critical. Although the electroshock procedure is straightforward, practice is required to stimulate animals in a reproducible fashion.

Dose

The most critical parameter in the epilepsy models that use chemical-induced seizures is the dose of the chemical convulsant compound. The doses described in this unit are generally correct, but investigators must conduct preliminary experiments to determine the best dose to use in their laboratory conditions. This is especially true when setting up the low-dose PTZ (85 mg/kg, s.c.) test in which the majority of mice should have clonic convulsions, and only a few should have tonic convulsions.

Troubleshooting

Table 5.22.7 describes some common problems encountered with the procedures described in this unit, along with explanations of possible causes and suggestions for how to overcome the problems.

Anticipated Results

Tables 5.22.1, 5.22.2 , 5.22.3, and 5.22.4 show how raw data and statistical results are tabulated for the MES, threshold MES, PTZ (120 mg/kg), and PTZ (85 mg/kg) protocols. The data sheets for the BIC, PIC, and STR

Table 5.22.7 Troubleshooting Guide for Electroshock and Chemical Induced Convulsions in the Mouse

Problem	Possible cause	Solution
No convulsions, arcing between electrodes and eyes	Poor electrical contact with corneas	Check if corneas contact stimulating electrodes, adjust electrode spacing.
		Hold corneas firmly against corneal electrodes.
		Do not release mouse before stimulation is complete.
		Apply more electrode gel.
		Check if electroshock apparatus is delivering current, using troubleshooting procedure in apparatus manual.
No hind-limb tonic extensor convulsion	Weak electrical stimulation	Adjust current intensity and/or duration until tonic convulsion occurs.
PTZ induces tonic convulsions when only clonic convulsions are desired	Dose of PTZ too high	Recommended dose is 85 mg/kg, subcutaneously for clonic convulsions, but optimum dose may vary with mouse strain. Decrease dose until at least 90% of mice have clonic convulsions.
PTZ induces clonic convulsion in <90% of mice	Dose of PTZ too low	Recommended dose is 85 mg/kg, subcutaneously for clonic convulsions, but optimum dose may vary with mouse strain. Increase dose until at least 90% of mice have clonic convulsions.
Bicuculline, picrotoxin, or strychnine fails to produce convulsions in at least 90% of vehicle-treated mice	Dose too low	Increase dose.

Table 5.22.8 Analysis of The Effects of Valproic Acid on Pentylenetetrazol (PTZ) (85 mg/kg)-Induced Seizures in Mice by One-Way Analysis of Variance and Dunnett's Multiple Comparison Test

Treatment	mg/kg, i.p. injected 15 min before PTZ	Latency in sec, mean (±SEM)[a]
Vehicle	—	479 (159)
Valproic acid	30	214 (42)
	100	631 (159)
	200	1058 (221)[b]
	300	1800[b]

[a]SEM, standard error of the mean.
[b]$p < 0.05$ versus vehicle.

Animal Models of Disease

typically resemble that of PTZ (85 mg/kg). A table for PTZ infusion data would include columns for the volume of PTZ infused to initiate each seizure event and latency to each seizure event. Investigators may also want to include a column for the weight of each mouse in the dose group. Table 5.22.5 shows the typical tabulated presentation of data from a PTZ infusion experiment. Table 5.22.8 shows the application of the one-way analysis of variance and post hoc test applied to the analysis of valproic acid data presented in Table 5.22.4. The Student's *t*-test, analysis of variance, and various post hoc tests, Fisher's exact test, and linear regression statistics are available in many statistics packages for the personal computer.

Time Considerations

The time required for anticonvulsant experiments includes the time required to prepare the solutions of chemical convulsants and test substances, the time needed for the staggered administration of the test substance and reference compound (for example, ten mice in a group with each mouse injected every 5 min for a total of 50 min), the corresponding application of the MES or injection of the chemical convulsant (for example, at 30 min after injection of the test substance), and the time required for observation of the mice after MES (5 min), or after the administration of a chemical convulsant (15 to 30 min). This assumes that the cages and other apparatus are already set up for the experiment.

The MES and PTZ tests can be used to screen test substances rapidly for anticonvulsant activity or to determine an ED_{50} value. Only one dose of the test substance is generally evaluated in a rapid screen. This approach is especially efficient when testing compounds from a series of compounds known to have anticonvulsant activity, and a single screening dose can be selected for the series.

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